AD_____

Award Number: W81XWH-06-1-0176

TITLE: Neurofibromatosis and the Painful Neuroma

PRINCIPAL INVESTIGATOR: Allan Joel Belzberg, M.D.

CONTRACTING ORGANIZATION: Johns Hopkins University
Baltimore MD 21218-2686

REPORT DATE: January 2008

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	**2. REPORT TYPE**	**3. DATES COVERED** *(From - To)*
01-01-2008	Annual	01 Jan 07 – 31 Dec 08

4. TITLE AND SUBTITLE
Neurofibromatosis and the Painful Neuroma

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-06-1-0176

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Allan Joel Belzberg, M.D.

E-Mail: belzberg@jhu.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Johns Hopkins University
Baltimore MD 21218-2686

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
Pain is a common and distressing symptom that impacts the quality of life of many patients with neurofibromatosis. The pain is often due to the formation of a neuroma. To understand better how neuromas cause pain and what treatments may be provided, we have developed an animal model of a painful neuroma. The tibial neuroma transposition (TNT) model has been confirmed as a model of neuropathic pain. The TNT model has been established as reliable and valid (Specific Aim 1). In the TNT model, the neuroma test-site mechanosensitivity is dependent on neural input from the tibial neuroma. In the TNT model, hindpaw mechanical hyperalgesia is independent of input from the tibial neuroma. We have altered the formation of a neuroma by applying a toxin that Pain is a common and distressing symptom that impacts the quality of life of many patients with neurofibromatosis. The pain is retrogradely transported (suicide transport) leading to neuronal death and axonal death (Specific Aim 2). This technique is now being refined using target-specific toxins and examining subsequent pain behaviour (Specific aim 3).

15. SUBJECT TERMS
Neurofibromatosis, Neuroma, Neuropathic Pain

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			**19b. TELEPHONE NUMBER** *(include area code)*
U	U	U	UU	28	USAMRMC

Table of Contents

Page

INTRODUCTION

A percentage of NF1 patients may experience an increase in pain after surgical removal of a neurofibroma. This pain is due to the formation of a painful neuroma, a jumbled mass of nerve fibers and connective tissues, at the cut end of the nerve. Palpating the tissue overlying a neuroma evokes paresthesias/dysasethesias in the distribution of the injured nerve. Surgical resection of the neuroma may provide relief, but the pain often recurs following the inevitable evolution of a new neuroma at the nerve end. Previous animal models of neuropathic pain have focused on the mechanical hyperalgesia and allodynia that develops at a location distant from the site of injury and not on the pain from direct stimulation of the neuroma. We describe a new animal model of neuroma pain, the tibial neuroma transposition (TNT) model, in which the neuroma is located in a position that is accessible to mechanical testing and outside of the innervation territory of the injured nerve. This allows testing of pain in response to mechanical stimulation of the neuroma (which we call neuroma tenderness) independent of pain due to mechanical hyperalgesia. Mechanical stimulation of the neuroma produced a profound withdrawal behavior that could be distinguished from the hyperalgesia that developed on the hindpaw. The ultimate objective of this research is to prevent reformation of a painful Neuroma by using suicide transport of neuronal toxins.

BODY

We will present a summary of our efforts that represent 1, research based directly on the specific aims of the grant and 2, outgrowth research to improve methodology in this work and increase our understanding of the patho-physiology underlying neuropathic pain.

1) Specific Aim Directed Research

In year one, we firmly established the TNT model with the addition of sufficient animal numbers to our preliminary work to produce a reliable, statistical and publishable result. We then completed our first specific aim by demonstrating that blocking neural input from the neuroma to the CNS reversed the pain behavior produced by the TNT model. In year 2 we have been experimenting with a variety of neural toxins to prevent neuroma formation through retrograde transport and cell death.

<u>Retrograde Transport Experiments</u>

Our initial experiments focused on using OX7-SAP (Specific Aim 2). This toxin consists of the mitogen recognizing complex monoclonal antibody, OX7, specific for the rat Thy 1 coupled to saporin, a ribosome inactivating protein. The target antigen, rat Thy 1, is present on all adult neuronal cells. As Thy 1 is present on neurons and essentially absent from other tissues, the expectation was that injection of OX7-SAP into the neuroma would selectively destroy the neurons terminating there. The OX7-SAP experiments did not provide consistent outcomes. One possible explanation is that some antigenic structures of neurons are down-regulated after injury. A recently published study indicates that Thy 1 is transiently decreased in DRG neurons after sciatic nerve crush. It is important to note that although consistent with our findings, this information was not available at the commencement of our studies. Nonetheless, it was decided that the most effective next step was to examine the efficacy of a less selective suicide transport molecule: Ricin.

Subsequent work has focused on establishing a proof of principal for suicide transport. Ricin is a neurotoxic lectin, derived from castor beans, that binds to certain oligosaccharides on the cell surface. After binding, it undergoes endocytosis and is axoplasmically transported to the cell body where it causes ribosome inactivation, inhibition of protein synthesis, and ultimately cell death. An intraneural injection of ricin causes a loss of neurons in the corresponding DRG, anterior motor horn region, and complete degeneration (Wallerian) of both myelinated and unmyelinated axons in the peripheral nerve, central processes, and terminals. We decided to use Ricin injections as a proof of principal experiment for the concept of preventing painful neuroma formation as it is has a more robust and less specific effect to OX7-SAP.

In discussion with various experts in the field of neural toxins and retrograde transport we identified Wheat Germ Agglutinin (WGA) coupled to Saporin as an interesting neuronal toxin. This toxin will bind

preferentially to small neuronal fibers, ones usually associated with pain transmission. We decided to pursue additional experiments and determine if this toxin would produce neuronal cell death and Wallerian degeneration and prevent the development of a painful neuroma.

Method

Establishing the TNT model

The posterior tibial nerve was exposed from approximately 8 mm proximal to the calcaneal branch to 1 mm distal to the plantar nerve bifurcation. The integrity of the calcaneal branch was preserved while it was dissected free from the main trunk of the tibial nerve. Just proximal to the plantar bifurcation, the tibial nerve was tightly ligated with 6-0 silk and sharply transected with scissors. Using a blunt glass probe, a subcutaneous tunnel was burrowed from the medial incision site to the lateral aspect of the hind limb. A 1.5 mm diameter plastic tube with a longitudinal slit in one wall was placed in the tunnel. The needle-baring end of the suture used to ligate the tibial nerve was passed through the plastic tube and pushed through the skin at a location 8-10 mm superior to the lateral malleolus. The plastic tube was then removed from the tunnel. The suture was gently pulled to advance the tibial nerve stump through the subcutaneous tunnel, until it was flush with the inner surface of the skin of the lateral hind limb. The suture was then cut flush with the skin. The incision was closed with running 6-0 silk sutures. The subsequent neuroma was located in a lateral position that was easily accessible for mechanical testing. The suture material could be viewed just below the skin surface and provided a target for mechanical testing.

Behavioral Testing

The rats were tested three times preoperatively and several times during the postoperative period. The animals were placed in individual transparent plastic cages on top of an elevated wire mesh stage that allowed access to the plantar surface of the paw. A 2.5 x 20 cm window at the bottom of the sidewalls of the cages permitted application of von Frey filaments to the ankle region. The animals were allowed to acclimate to the testing environment for 20-30 minutes before testing began

Neuroma Tenderness

The suture tied to the distal end of the tibial nerve or connective tissue was visible through the skin and served as the target for mechanical stimuli. An analogous site served as the target on the contralateral hindlimb. A trial consisted of a train of five applications of a von Frey filament (150 mN for 1-2 s) with an interstimulus interval of 1 s. If the animal responded to any of the five applications, the trial was terminated. A positive response was defined as a slow withdrawal of the hindpaw, or rapid withdrawal with vocalization, licking, or shaking.

A grading system to qualitatively evaluate behavioral responses was used. Each trial was assigned a response grade ranging from 0 to 2 based on the animal's response. A grade of 0 indicated that the animal did not respond during a given trial. A grade 1 response represented a slow withdrawal of the paw. A grade 2 response was defined as a brisk withdrawal or shaking, licking, or vocalization. The Withdrawal Score was defined as the sum of response grades for five trials and ranged from 0 to 10. (See our recently published manuscript that is included in this report for additional details of the testing procedure)

Nerve Injection

Just proximal to the plantar bifurcation, the tibial nerve is sharply transected with scissors. Forceps are used to crush the nerve 2 mm proximal to the cut end of the nerve. A glass micropipette connected to a Hamilton microliter syringe pump system (Hamilton Company, 4970 Energy Way, Reno, Nevada USA) is carefully inserted into the center of the proximal stump of the tibial nerve and advanced to the region of the nerve that was crushed. Then, 2 µl volume of toxin or control is injected over 20 minutes. An indicator dye (fast green) was included with the drug to allow us to verify the drug does not leak out of the nerve during the injection procedure. If a substantial amount of dye leaked out, we excluded the animal from the subsequent analysis. After drawing out the micropipette, the stump of the tibial nerve is tightly ligated with 6-0 silk, and then transposed to the lateral hindlimb (as described above for TNT model surgery).

Histopathology

Animals were deeply anesthetized with 10% isoflurane and received a transcardiac perfusion with 0.1M PBS (pH 7.4), followed by 4.0% paraformaldehyde solution in Sorenson's buffered solution (pH 7.4). All surgical lesions were grossly confirmed. The tibial nerve was inspected for the presence of a neuroma (when appropriate). The nerves to be studied (sciatic, tibial, calcaneal, peroneal) were harvested. Relevant spinal cord and DRG were harvested. Serial sections were taken beginning at the distal nerve end and proceeding proximal. Peripheral nerves and dorsal/ventral roots. For plastic section, the specimens are fixed in 2% glutaraldehyde in buffered cacodylate. Serial dehydration through graded ethyl alcohol solution is performed over 24 hours. The specimens are then post-fixed in 2% osmium tetroxide and embedded in Epon. Plastic embedded tissue will produce 1 micron sections to be stained with toluidine blue. Paraffin sections are post-fixed for 48 hours in 4% paraformaldehyde then transferred to PBS and kept at 4°C until sectioning. Paraffin sections will produce 12 micron sections to be stained with hematoxylin and eosin and alternatively examined unstained. DRG and spinal cord. Paraffin sections are post-fixed for 4-6 hours in 4% paraformaldehyde then transferred to PBS and kept at 4°C until sectioning. Paraffin sections will produce 12 micron sections to be stained with hematoxylin and eosin and alternatively examined unstained.

Experimental Design

The experiments examining retrograde transport of a neural toxin and painful neuroma prevention were initially performed in an open label format to determine drug dosing and pharmacological effect. All subsequent studies included blinding of the examiner. The retrogradely transported toxins that we have examined included OX7-SAP, WGA-SAP, and Ricin.

Results

OX7-SAP

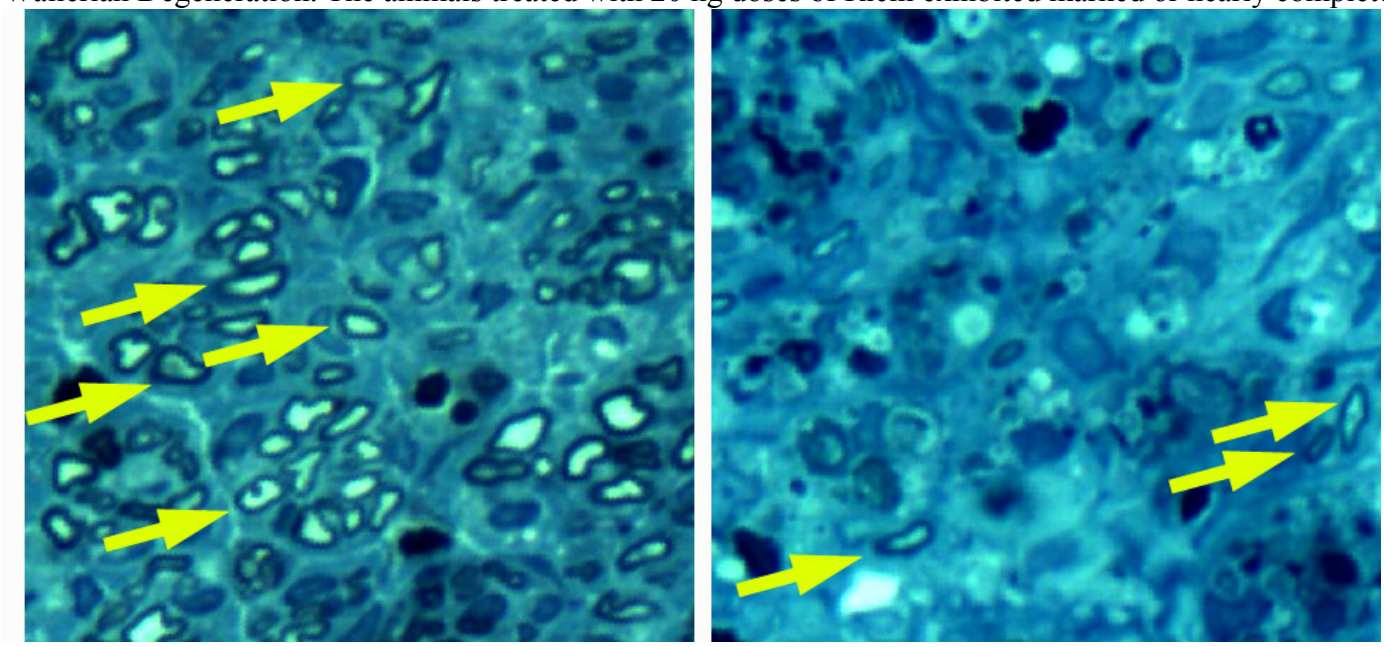
In our initial open labeled studies, we used OX7-SAP doses up to 2 ug in 2 ul. It was initially surprising to find that even the highest dose (2 ug, see Figure 1 in Supporting Data) did not lead to a decrease in neuroma tenderness. Given that the Thy-1 receptor is not found in human and the lack of a behavioral result, we decided to try other toxins instead of pursuing OX7-SAP. As previously stated, a recent publication has documented the down regulation of the receptor after axotomy providing a possible explanation for the lack of experimental effect.

Ricin

Our initial open labeled studies used doses ranging from 0.5 ng to 500 ng of Ricin in 2 ul. In these studies, we saw a decrease in the behavioral response to neuroma stimulation at some of the middle doses. At the higher doses (≥ 50 ng) we saw localized tissue necrosis overlying the neuroma site that became more pronounced at higher doses. We therefore chose to investigate the following doses in our blinded and controlled study (1, 5, 10, 20 ng). The behavioral results from these data are shown in Figure 2 (see Supporting Data). In the top panel of Figure 2, the behavioral responses to von Frey stimulation of the neuroma site are plotted as a function of time after the lesion. Each curve corresponds to a different dose of ricin. In bottom panel of Figure 2, the dose response function for ricin is plotted. For these data, we determined the average behavioral response after the ricin administration (i.e., average response from day 9 to 35). Ricin led to a significant reduction in the behavioral response at both the 10 ng and 20 ng dose.

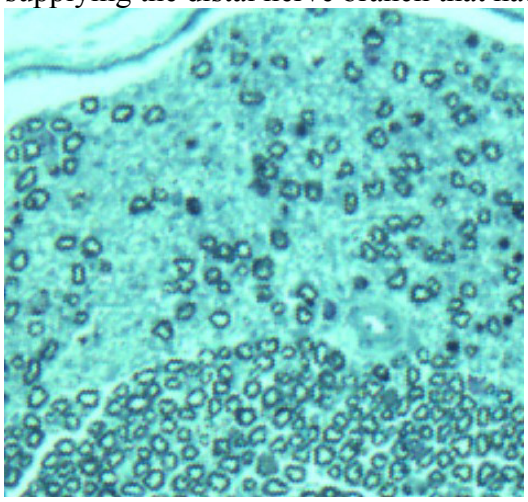
On histology review, we found that the highest doses of Ricin (≥ 50 ng) produced a nearly complete elimination of myelinated axons from the neuroma itself. Examination of more proximal nerve segments demonstrated segmental Wallerian Degeneration as would be expected for a toxin producing cell death. The lowest doses resulted in a minimal reduction in myelinated axons in the neuroma itself with some proximal

Wallerian Degeneration. The animals treated with 20 ng doses of Ricin exhibited marked or nearly complete



elimination of myelinated axons from the neuroma itself. An example of this is shown in the figure which compares the appearance of an neuroma exposed to 1 ng of Ricin (nearly normal neuroma with many myelinated axons, examples are indicated by the yellow arrows but dozens are present) to the appearance of a neuroma treated with 20 ng of Ricin (nearly all myelinated axons are degenerated, three axons are indicated by yellow arrows).

Additional observations were made of the proximal nerves and the dorsal root ganglia, where the cell bodies of the C-fiber axons are located and the spinal cord. In the proximal nerves, there was focal Wallerian degeneration, that was focal within the nerve indicating that this was due to proximal degeneration of axons supplying the distal nerve branch that had been treated with Ricin. This is illustrated in the figure at left where the normal density of axons in an untreated branch of the nerve is seen in the lower part of the figure, the depleted density of axons due to treatment is seen in the upper portion of the figure. In the DRG, there was evidence of single, isolated neurons undergoing phagocytosis by leukocytes; strikingly, adjacent neurons were completely normal in appearance as gauged by the H&E staining. These findings were consistent with the toxin working via a dose-dependent mechanism and inducing neuronal death by a suicide transport mechanism.



Taken together, these data demonstrate the proof-of-concept that injection of suicide transport molecules can eliminate the myelinated nerve endings from a neuroma and lead to a significant reduction of neuroma tenderness.

Wheat Germ Agglutinin - SAP

In our open labeled studies we used doses of WGA-SAP ranging from 5 to 200 ng. None of these doses led to a significant decrease in the behavioral responses to neuroma stimulation.

On histology review, we observed dose dependent effect at the level of the neuroma. This effect consisted of a decrease in the number of unmyelinated axons and an increase in the number of regenerative clusters. More proximal portions of the nerve were evaluated by both plastic section analysis and selected nerves by paraffin section. The plastic section analysis of the proximal nerves indicated that, except at the highest dose, the myelinated axons were essentially normal in appearance with a very small number of myelinated axons

undergoing Wallerian Degeneration. Aside from the preservation of myelinated axons, there appeared to be a decrease in C-fibers in segments of the nerve however, given the random distribution of C-fibers in the normal nerve this was difficult to assess fully. There was however an increase in nuclear number, consistent with increased numbers of Schwann cells in some segments of the proximal nerve. This was confirmed by examination of the paraffin sections stained with H&E.

The histology observations were consistent with the degeneration of C-fiber axons which, being unmyelinated, leave few traces after undergoing abrupt degeneration. The one remnant of this process is typically an increased number of Schwann cells as observed in these nerves. These findings are consistent with a highly selective destruction of small fiber axons and the associated neurons. This is strongly supportive of successful targeting of the WGA-SAP to the intended target.

Putting together the behavioral and histology results, the data demonstrate that elimination of most unmyelinated fiber innervation of the neuroma does not eliminate neuroma tenderness. This has led to our current hypothesis that the neuroma tenderness is due to activity in myelinated fibers. This is consistent with the clinical observation that tapping on a neuroma leads to sharp, "electric" pain that is immediately perceived (i.e., faster than would be conducted by activation of unmyelinated fibers). To pursue this hypothesis, we plan to use a toxin that is specific to myelinated fibers. Initially we will use CTB-SAP.

2) Outgrowth Research

Anti-ganglioside Antibodies

Dr. Kazim Sheikh from Johns Hopkins Department of Neurology has demonstrated that the systemic administration of a monoclonal anti-ganglioside antibody leads to profound inhibition of axon sprouting in a sciatic nerve crush model. He has obtained funding from the Blaustein Pain Foundation to explore the hypothesis that this technique could be used to alter pain from nerve injury. Part of the basis of the grant is a collaboration with our group in that we would provide him the TNT animal models and behavioral testing technique.

Gangliosides, the target antigens of anti-ganglioside antibodies (Abs), are major cell surface determinants and the predominant sialoglycoconjugates in the mammalian nervous system. GD1a and GT1b are the two major gangliosides in the growth cones of regenerating axons. Abs with GD1a specificity can act as inhibitory cues for growth cones .

Initial experiments determined a pharmacologic effect on axonal sprouting. These were used to determine a dose for treating animals in whom the TNT model was being established. The average behavioral response to mechanical stimulation at the neuroma site was significantly lower ($p<0.01$) for the nerves in which the antibody was injected (5.2 =/- 0.7, n=4) compared to the control nerves that were injected with mouse serum (9.0 +/- 0.3, n=4).

While this is very preliminary data, it does appear that the administration of an Abs may prevent painful Neuroma formation. We will continue these collaborative studies with carefully controlled and blinded experiments.

KEY RESEARCH ACCOMPLISHMENTS

- The formation of a neuroma subsequent to axotomy can be altered by using retrograde transport of a neural toxin in the proximal stump.

- Neuroma test-site mechanosensitivity can be altered by retrograde transport of a neural toxin.

- The pain behavior associated with neuroma formation may not be dependent on ongoing activity in small fiber neurons (C-fibers, A-delta fibers).

- The administration of a systemic anti-ganglioside antibody may prevent axonal sprouting in a neuroma and the thus prevent the development of neuropathic pain secondary to Neuroma mechanosensitivity.

REPORTABLE OUTCOMES

The tibial neuroma transposition (TNT) model of neuroma pain and hyperalgesia.
M. Dorsi, L. Chen, B. Murinson, E. Pogatzki-Zahn, R. Meyer, A. Belzberg
Pain, Volume 134, Issue 3, Pages 320-334

CONCLUSION

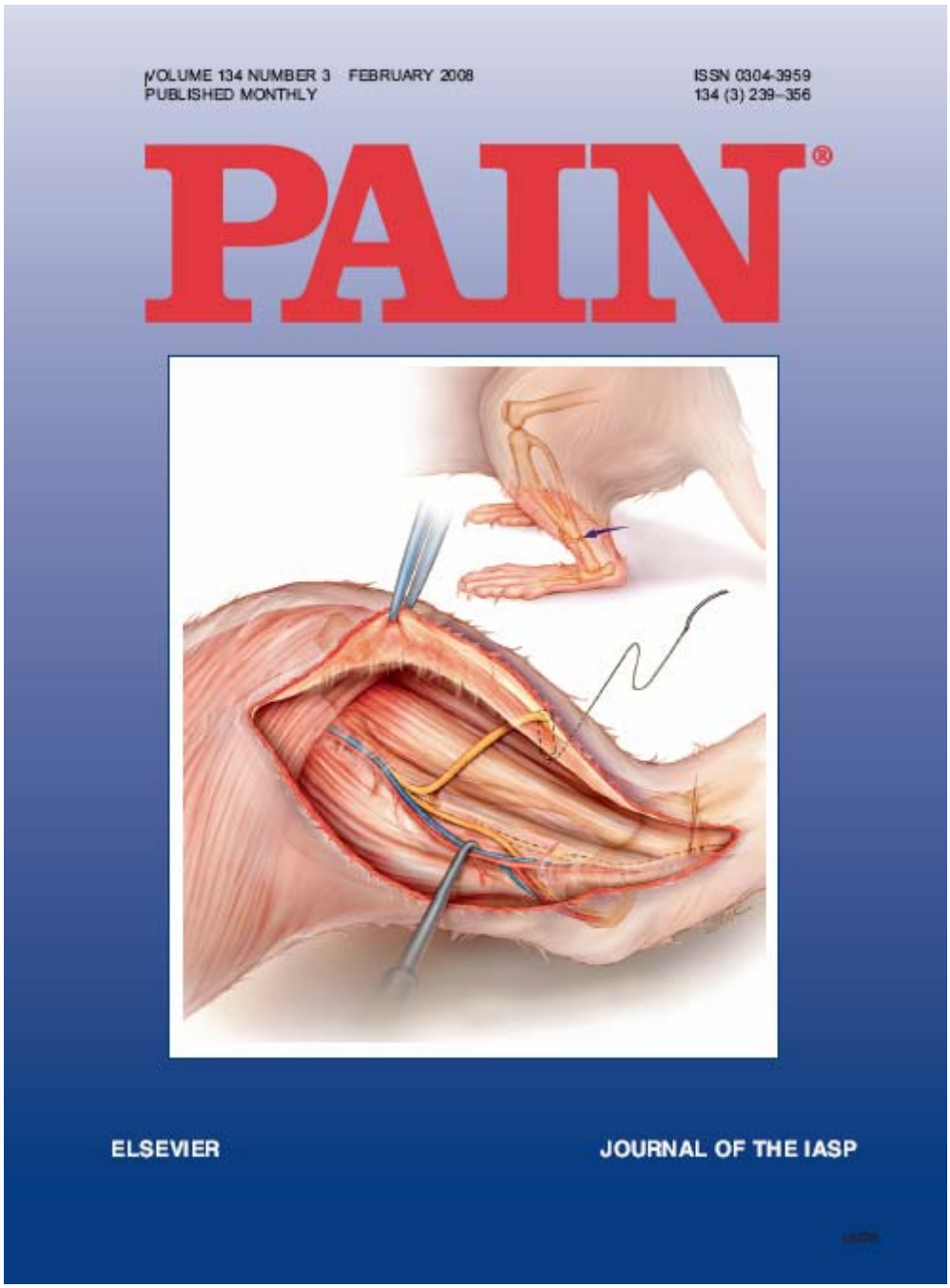
We have now completed the first and second of three specific aims.
The pain behavior displayed by the animal results from mechanical stimulation of the neuroma, a phenomenon commonly seen in patients with painful neuroma. The tibial neuroma transposition (TNT) model provides the scientific community an animal model of neuroma pain.

The application of Ricin to the nerve will result in retrograde transport of the neural toxin and axonal degeneration. There is a dose dependent loss of axons and prevention of neuroma formation.
The application of Wheat Germ Agglutinin – SAP to a nerve will result in retrograde transport of the neural toxin and loss of small fiber axons. In preliminary experiments, the loss of these "pain fibers" did not result in a loss of pain behavior. This phenomenon will be further explored.

We will now proceed with the third and final specific aim. We will alter neuroma formation using retrograde transport of a variety of neural toxins and carefully determine the effects on pain behavior. Specifically, we will determine if we can both prevent painful neuromas from forming and reverse pain behavior by treating an existing neuroma.

We will pursue the use of anti-ganglioside antibody in preventing neuroma formation through a collaboration with Dr. Kazim Sheikh from Johns Hopkins Neurology.

APPENDICES


VOLUME 134 NUMBER 3 FEBRUARY 2008
PUBLISHED MONTHLY
ISSN 0304-3959
134 (3) 239–356
PAIN®
ELSEVIER
JOURNAL OF THE IASP

Pain 134 (2008) 320–334

PAIN

www.elsevier.com/locate/pain

The tibial neuroma transposition (TNT) model of neuroma pain and hyperalgesia

Michael J. Dorsi [a,1], Lun Chen [a,1], Beth B. Murinson [b], Esther M. Pogatzki-Zahn [a,d], Richard A. Meyer [a,c], Allan J. Belzberg [a,*]

[a] Department of Neurosurgery, The Johns Hopkins University, School of Medicine, 5-181 Meyer Building, Baltimore, MD 21287, USA
[b] Department of Neurology, The Johns Hopkins University, School of Medicine, 5-181 Meyer Building, Baltimore, MD 21287, USA
[c] Applied Physics Laboratory, Johns Hopkins University, Laurel, MD, USA
[d] Department of Anesthesiology and Intensive Care, University of Muenster, Muenster, Germany

Received 30 January 2007; received in revised form 22 May 2007; accepted 25 June 2007

Abstract

Peripheral nerve injury may lead to the formation of a painful neuroma. In patients, palpating the tissue overlying a neuroma evokes paraesthesias/dysaesthesias in the distribution of the injured nerve. Previous animal models of neuropathic pain have focused on the mechanical hyperalgesia and allodynia that develops at a location distant from the site of injury and not on the pain from direct stimulation of the neuroma. We describe a new animal model of neuroma pain in which the neuroma was located in a position that is accessible to mechanical testing and outside of the innervation territory of the injured nerve. This allowed testing of pain in response to mechanical stimulation of the neuroma (which we call neuroma tenderness) independent of pain due to mechanical hyperalgesia. In the tibial neuroma transposition (TNT) model, the posterior tibial nerve was ligated and transected in the foot just proximal to the plantar bifurcation. Using a subcutaneous tunnel, the end of the ligated nerve was positioned just superior to the lateral malleolus. Mechanical stimulation of the neuroma produced a profound withdrawal behavior that could be distinguished from the hyperalgesia that developed on the hind paw. The neuroma tenderness (but not the hyperalgesia) was reversed by local lidocaine injection and by proximal transection of the tibial nerve. Afferents originating from the neuroma exhibited spontaneous activity and responses to mechanical stimulation of the neuroma. The TNT model provides a useful tool to investigate the differential mechanisms underlying the neuroma tenderness and mechanical hyperalgesia associated with neuropathic pain.

Keywords: Neuroma; Neuropathic pain; Hyperalgesia; Nerve injury; Central sensitization; Allodynia; Neurofibroma

1. Introduction

Painful neuromas can arise from peripheral nerve injuries such as trauma, amputation, nerve biopsy, or resection of a neurofibroma. Patients experience tenderness to palpation of the skin overlying the neuroma, spontaneous burning pain, and allodynia and hyperalgesia in the distribution of the injured nerve. Despite advances in our understanding of neuropathic pain, providing adequate pain relief for these patients remains a clinical challenge. Unfortunately, a substantial proportion of patients develop pain that is refractory to contemporary pharmacological, psychological, and surgical intervention and endure significant disability. Therefore, research is needed to further increase our understanding of neuropathic pain and to develop novel therapies.

A number of animal models that involve traumatic nerve injuries have been developed to study neuropathic

* Corresponding author. Tel.: +1 410 955 2058; fax: +1 410 955 1032.
E-mail address: abelzber@jhmi.edu (A.J. Belzberg).
[1] The first two authors contributed equally to this study.

0304-3959/$32.00
doi:10.1016/j.pain.2007.06.030

pain including sciatic nerve transection (Wall et al., 1979), chronic constriction injury (Bennett and Xie, 1988), partial sciatic nerve ligation (Seltzer et al., 1990), spinal nerve ligation (Kim and Chung, 1992), and spared nerve injury (Decosterd and Woolf, 2000). These contemporary models of neuropathic pain have greatly expanded our understanding of the mechanisms underlying neuropathic pain. However, several characteristics of these models limit their usefulness in studying neuroma pain. First, the observed behavioral changes are evoked by stimuli applied to the hindpaw at a location distant from the site of injury. There is currently no model measuring the effect of directly applying stimuli to the neuroma. Second, there is mounting evidence that hyperalgesia in the existing models can develop in the hindpaw independent of input from injured afferents and thus independent of the neuroma (Eschenfelder et al., 2000; Li et al., 2000). Further, hyperalgesia may develop following lesions that do not involve injury to afferent fibers (e.g., ventral rhizotomy) (Li et al., 2000; Sheth et al., 2002) or the formation of a neuroma (Eschenfelder et al., 2000; Sheth et al., 2002). These findings suggest that ectopic activity originating from a neuroma is not necessary for development of hyperalgesia.

We aimed to develop an animal model of neuroma pain. An ideal model would produce robust, severe, and lasting behavioral changes resembling those seen in patients with painful neuromas (i.e., ongoing pain sensations, pain evoked by palpation of the skin overlying the neuroma, and hyperalgesia in the distribution of the injured nerve). We propose that distinct but overlapping pathophysiological mechanisms underlie the multiple pain phenomena produced by peripheral nerve injury.

We based our model on the clinical observation that mechanical stimuli applied to the skin overlying a neuroma produce paraesthesias or lancinating pain in the distribution of the nerve (Hoffman-Tinel sign). It is believed that this clinical sign is indicative of ectopic mechanosensitivity of injured or regenerating afferent fibers. We hypothesize that mechanical stimuli applied to the skin overlying a neuroma in a rat will elicit a similar sensation and provoke foot withdrawal. Further, we hypothesize that mechanical hyperalgesia will develop in the cutaneous distribution of the injured peripheral nerve. Thus a peripheral nerve injury model was created that would permit the independent study of these two distinct pain behaviors.

2. Methods

2.1. Experimental animals

Eighty male Sprague–Dawley rats (Harlan, Indianapolis, IN) weighing 200–250 g were studied. Two to four animals were placed in plastic cages with sawdust bedding, housed in a climate controlled room under a 14/10 light/dark cycle, and provided food and water ad libitum. The animals were acclimatized under these conditions for at least a week before the initiation of experimentation. The Johns Hopkins University Animal Care and Use Committee approved the testing and surgical protocol.

2.2. Surgical procedures

The animals were randomly assigned to surgical groups for each experiment. For all surgical procedures, deep anesthesia was maintained throughout surgery with 2% isoflurane. All incisions were closed with running 6-0 silk sutures. All procedures were performed with a dissection microscope. At the conclusion of each experiment, all lesions were confirmed at autopsy.

2.2.1. Tibial neuroma transposition (TNT) model

The objective of the tibial neuroma transposition surgery was to produce a neuroma that was located in a position that was accessible for mechanical testing and that was outside of the innervation territory of the injured nerve. This allowed testing of pain in response to mechanical stimulation of the neuroma (which we call "neuroma tenderness") independent of pain due to hyperalgesia.

Our decision to use the tibial nerve was also based on the following factors: (1) The tibial nerve innervates the plantar surface of the hindlimb. The expected behavioral response to tibial neuroma stimulation would be hindlimb withdrawal. This behavior is easy to quantify and commonly used in most contemporary models of neuropathic pain. With experience we were able to increase the specificity of behavioral testing by scoring the intensity of hindpaw withdrawal. (2) The tibial nerve is a mixed nerve comprised of both sensory and motor nerve fibers. Thus, a tibial neuroma would be expected to develop electrophysiological properties similar to those demonstrated in other mixed nerve neuroma preparations (e.g. spinal nerves and sciatic nerve).

As illustrated in Fig. 1 (see also Fig. 3A), the posterior tibial nerve was exposed from approximately 8 mm proximal to the calcaneal branch to 1 mm distal to the plantar nerve bifurcation. The integrity of the calcaneal branch was preserved while it was dissected free from the main trunk of the tibial nerve. Just proximal to the plantar bifurcation, the tibial nerve was tightly ligated with 6-0 silk and sharply transected with scissors.

Using a blunt glass probe, a subcutaneous tunnel was burrowed from the medial incision site to the lateral aspect of the hindlimb. A 1.5 mm diameter plastic tube with a longitudinal slit in one wall was placed in the tunnel. The needle-bearing end of the suture used to ligate the tibial nerve was passed through the plastic tube and pushed through the skin at a location 8–10 mm superior to the lateral malleolus. The plastic tube was then removed from the tunnel. The suture was gently pulled to advance the tibial nerve stump through the subcutaneous tunnel, until it was flush with the inner surface of the skin of the lateral hind limb. The suture was then cut flush with the skin. The subsequent neuroma was located in a lateral position that was easily accessible for mechanical testing (Fig. 1, top). The suture material could be viewed just below the skin surface and provided a target for mechanical testing.

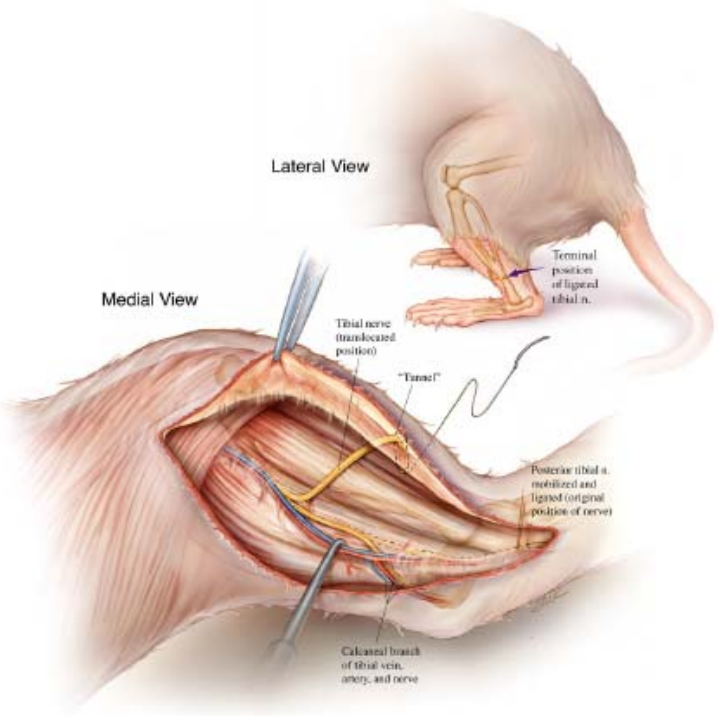


Fig. 1. The tibial neuroma transposition (TNT) model. Schematic depicting TNT model surgery. The distal tibial nerve in the foot is dissected free of adjacent tissue, ligated with a suture, and cut. The needle from the suture is passed through a subcutaneous tunnel to the lateral aspect of the hindlimb where it is pushed through the skin. The nerve is drawn into the tunnel until the ligature is adjacent to the skin. The suture is cut, and the incision closed (artwork by Ian Suk, Johns Hopkins University).

2.2.2. Sham surgery (S)

The tibial nerve was dissected as described above and left intact. A subcutaneous tunnel was formed as described above. A small piece of connective tissue was ligated and passed through the subcutaneous tunnel in the method described above (Fig. 3B).

2.2.3. Tibial neuroma with no transposition (TNT-nT)

The tibial nerve was dissected, ligated, and transected as described above for the TNT model surgery, but not transposed. A subcutaneous tunnel was formed and connective tissue was ligated and passed through to the lateral hindlimb as described above (Fig. 3C).

2.2.4. Tibial neuroma transposition with simultaneous proximal transection (TNT-sPT)

The TNT model surgery was performed as described above. Once the nerve stump was in place on the lateral aspect of the foot, the tibial nerve was sharply transected with scissors at the proximal entrance of the subcutaneous tunnel (Fig. 3D).

2.2.5. Tibial neuroma transposition with delayed proximal transection (TNT-dPT)

The TNT model surgery was performed as described above. Twelve days after surgery, the animals were re-anesthetized, and the tibial nerve was dissected free. Three millimeters proximal to the tunnel entrance, the nerve was tightly ligated with 6-0 silk and a 2–3 mm segment of the tibial nerve distal to the ligature was removed (Fig. 3E). A 6-0 silk suture was then used to anchor adjacent connective tissue to close the entrance to the tunnel. For control animals, the nerve was exposed but not cut.

2.3. Behavioral testing with mechanical stimuli

To insure blinding, the experimenters doing the behavioral testing were blinded to the surgery of each animal, and the different surgical groups were tested concurrently. The rats were tested three times preoperatively and several times during the postoperative period. The animals were placed in individual transparent plastic cages on top of an elevated wire mesh stage

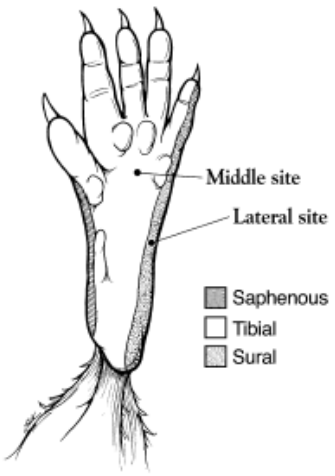


Fig. 2. Mechanical hyperalgesia testing sites. To test for mechanical hyperalgesia, von Frey probes were applied to lateral (sural distribution) or middle (tibial distribution) sites on the plantar surface of the paw. Nerve distributions were derived from Swett and Woolf (1985).

that allowed access to the plantar surface of the paw. A 2.5 × 20 cm window at the bottom of the sidewalls of the cages permitted application of von Frey filaments to the ankle region. The animals were allowed to acclimate to the testing environment for 20–30 min before testing began.

2.3.1. Neuroma tenderness

The suture tied to the distal end of the tibial nerve or connective tissue was visible through the skin and served as the target for mechanical stimuli. An analogous site served as the target on the contralateral hindlimb. A trial consisted of a train of five applications of a von Frey filament (150 mN for 1–2 s) with an interstimulus interval of 1 s. If the animal responded to any of the five applications, the trial was terminated. A positive response was defined as a slow withdrawal of the hindpaw, or rapid withdrawal with vocalization, licking, or shaking. Each testing session consisted of five trials to each hindlimb with an intertrial interval of about 2 min. The *Response Frequency* was defined as the percent of positive trials (i.e., 100 times the number of positive trials divided by five).

In later experiments, we implemented a grading system to qualitatively evaluate behavioral responses. Each trial was then assigned a response grade ranging from 0 to 2 based on the animal's response. A grade of 0 indicated that the animal did not respond during a given trial. A grade 1 response represented a slow withdrawal of the paw. A grade 2 response was defined as a brisk withdrawal or shaking, licking, or vocalization. The *Withdrawal Score* was defined as the sum of response grades for the five trials and ranged from 0 to 10.

Whether the response was specific to mechanical stimuli applied to the target site was evaluated in a small cohort of animals following TNT surgery. Testing was performed as described above, but in addition to the neuroma test site, stimuli were applied to skin overlying the tibial nerve 3 mm proximal to the neuroma, and the skin of the lateral hindlimb 3 mm and 5 mm inferior to the neuroma.

2.3.2. Hindpaw mechanical hyperalgesia

Mechanical withdrawal threshold to the application of a von Frey probe to the foot was measured by using the up-down method (Dixon, 1980). An ascending series of von Frey hairs of logarithmically incremental force (3.2, 5.2, 8.3, 15, 29, 44, 64, 94, and 160 mN) were applied to sites in the middle (tibial nerve distribution) and lateral (sural nerve distribution) aspect of the plantar surface of the left hindpaw (Fig. 2). Mechanical testing followed the procedure described by Ringkamp et al. (1999). Each von Frey hair was applied to the test area for about 2–3 s, with a 1–2 min interval between stimuli. A trial began with the application of the 15 mN von Frey probe to the left and right hindpaws of each animal. A positive response was defined as a rapid withdrawal and/or licking of the paw immediately upon application of the stimulus. Whenever a positive response to a stimulus occurred, the next smaller von Frey hair was applied, and whenever a negative response occurred, the next higher force was applied. The testing continued for five more stimuli after the first change in response occurred, and the pattern of responses was converted to a 50% von Frey threshold using the technique described by Dixon (1980). If the animal showed no response to the highest von Frey hair (160 mN), a von Frey threshold of 260 mN, corresponding to the next log increment in potential von Frey probes, was assigned to the threshold.

2.4. Lidocaine block

Nine weeks following TNT surgery, a cohort of eight rats displaying elevated behavioral response frequencies to mechanical stimulation of the neuroma and plantar mechanical hyperalgesia were randomly assigned to two interventional groups, local lidocaine injection or control lidocaine injection. In pairs, the animals were lightly anesthetized using 2% isoflurane. One animal received a 100 μl injection of 1% lidocaine with epinephrine to the neuroma target site marked by the suture on the tibial nerve. To control for systemic effects of lidocaine, the lidocaine/epinephrine was injected into the subcutaneous tissue overlying the lumbar spine of the other animal. Ten minutes after awakening from anesthesia, the animals were placed in cages on top of the testing stage as described above. Blinded behavioral testing of the neuroma and hindpaw was performed as described above immediately prior to lidocaine injection and three times after injection (15 min, 60 min, 120 min). Two days later, the animals were crossed over to the other treatment arm and the behavioral protocol was repeated.

2.5. Electrophysiological procedures

The rats were initially anesthetized with pentobarbital (50 mg/kg, intraperitoneal). Anesthesia was maintained by intravenous administration of pentobarbital (8–10 mg/kg/h) via the jugular vein. Heart rate was continuously monitored as an indicator of adequate anesthesia. A tracheotomy was

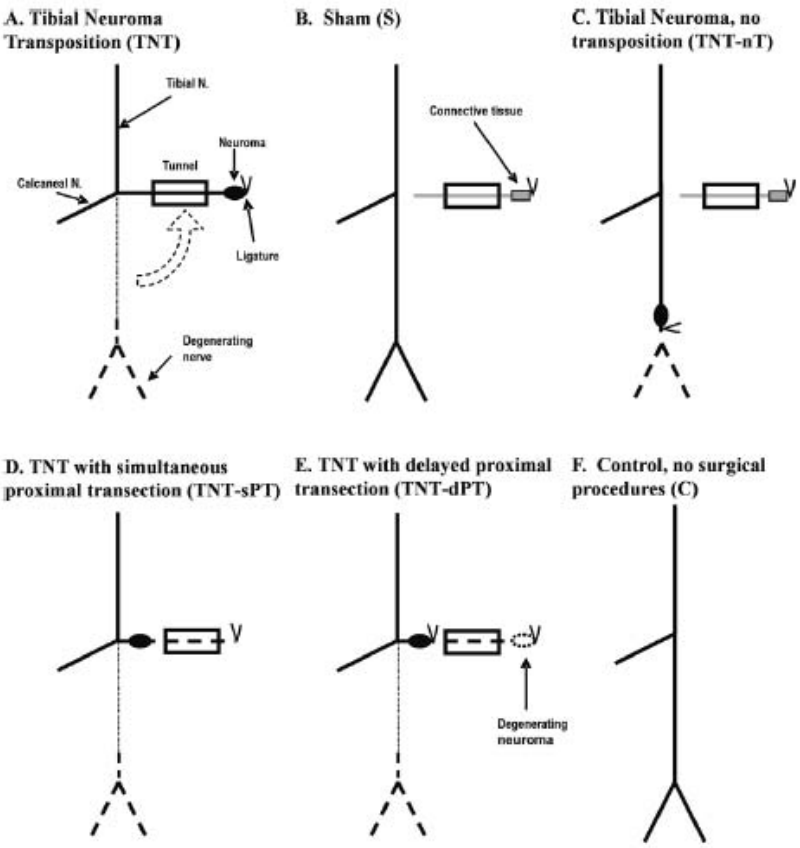


Fig. 3. Schematic of the different surgical groups.

performed, and animals were artificially ventilated to maintain expired pCO_2 to 40 mm Hg. Muscle paralysis was achieved by intravenous pancuronium bromide (1 mg/kg). Feedback-controlled, water-perfused heating pads were used to maintain core temperature (measured by a rectal probe) at 38 °C.

Electrophysiological recordings were made from the tibial nerve. Teased-fiber recording techniques were used as described previously (Campbell and Meyer, 1983). Briefly, a skin incision was made above the tibial nerve in the popliteal fossa, and the tibial nerve was exposed. The skin around the incision was used to form a pool by suturing the edges to a metal ring. The pool was filled with warm paraffin oil. A splitting platform was placed underneath the nerve at the proximal end, and a small silver wire which served as the recording electrode was positioned above the splitting platform. Small bundles were cut from the nerve, and teased into small filaments suitable for recording activity from single fibers. A stimulating electrode was placed under the nerve at the distal end of the incision, about 1.5 cm distal to the recording electrode. The stimulation electrode was used to deliver electrical pulses of variable strength to the nerve in order to count the number of A and C fibers on the recording electrode.

The neural signal was differentially amplified, filtered, and digitized at a rate of 25 kHz. A real-time computer-based data acquisition and processing system (DAPSYS, Brian Turnquist, Johns Hopkins University; for details, go to http://www.dapsys.net) was used to record neural activity. The software provided multiple window discriminators for real-time sorting of different action potential waveforms. All waveforms passing a selectable threshold level were saved for post hoc analysis.

Neural recordings were performed 100–120 days after the neuroma (or sham) surgery. After determining the number of fibers at the recording electrode that responded to electrical stimulation of the nerve, spontaneous activity was measured over a 5 min interval. A heat lamp was then applied to determine if the spontaneous activity originated from cold or warm

fibers. The skin over the neuroma site was stimulated with a von Frey probe (150 mN) and blunt pressure to determine whether mechanically sensitive fibers were present.

2.6. Histological procedures

Animals were euthanized by cardiac puncture under deep anesthesia and subsequently perfused with saline and 4% paraformaldehyde in Sorenson's buffered solution. Sections from the tibial nerve proximal and distal to all sites of ligation and transection, as well as the neuroma were harvested. The specimens were post-fixed in 2% osmium tetroxide and embedded in Epon. Sections (1 μm) were stained with toluidine blue.

2.7. Experimental design

Three separate groups of animals were included in the experiments described in this study.

2.7.1. Experiment group one

The aim of the initial experiment group was to demonstrate that the TNT model surgery led to the formation of a neuroma with characteristic electrophysiological and histological properties and also led to the development of a behavioral response that could be evoked by applying mechanical stimuli to the skin overlying the neuroma. The TNT surgery and the three different control procedures performed in experiment group one are illustrated in Fig. 3. The TNT model surgery was performed in eight animals (Fig. 3A). The three different control procedures were aimed at confirming that the pain behavior in response to palpating the ligature site was due to the neuroma formation. These control procedures were performed concurrently and were therefore also useful in blinding the experimenters. For eight animals, the TNT model surgery was performed, but the tibial nerve was simultaneously transected proximal to the tunnel (TNT-sPT, Fig. 3D). For eight additional animals, the tibial nerve was ligated and cut but not transposed to the lateral location (TNT-nT, Fig. 3C). Finally, the tibial nerve was exposed but not cut in eight sham animals (S, Fig. 3B). For all animals, a tunnel was created and a suture was placed under the skin. Behavioral testing for mechanical sensitivity at the neuroma test site was performed in all of the animals. In animals with the TNT, mapping of the behavioral response following application of stimuli at sites distant from the neuroma was also performed. Electrophysiological and histological studies were performed on a subset of these animals at the conclusion of the behavioral studies.

2.7.2. Experiment group two

The aim of the second experimental group was to investigate the effects of the TNT model on paw withdrawal thresholds to mechanical stimuli applied to the plantar surface of the hindpaw. In addition to TNT surgery ($N = 8$), two control groups were included in this experiment group. Eight animals underwent a sham procedure ("S", Fig. 3B), and eight animals did not undergo any surgical procedures ("C", Fig. 3F). All animals were tested for mechanical hyperalgesia in the hindpaw, as well as mechanical sensitivity at the lateral ankle (i.e., the neuroma test site). At the conclusion of eight weeks of behavioral testing, the eight animals that had received the TNT model surgery were selected for the lidocaine experimental protocol described above.

2.7.3. Experiment group three

The aim of the third experiment group was to determine if the behaviors provoked by applying mechanical stimuli to neuroma test site or plantar hindpaw depended on the presence of the neuroma at the lateral testing site. Twenty-four animals underwent TNT model surgery. Twenty animals that demonstrated robust neuroma tenderness and plantar hyperalgesia were selected and divided into two surgical groups. Ten days after the TNT surgery, 11 animals received a delayed proximal transection of the tibial nerve (TNT-dPT, Fig. 3E). To control the effects of re-exposing the tibial nerve, the tibial nerve was exposed but left intact in the remaining nine animals. All animals underwent additional behavioral testing of the neuroma test site and lateral aspect of the plantar hindpaw.

2.8. Statistical analysis

Since the behavioral scoring methods employed yield discrete prefixed values rather than a continuum, and since the data were not normally distributed because of the ceiling effects of a limited range of von Frey hairs, non-parametric tests were performed. Tests were performed to analyze the variance between testing days (Friedman ANOVA for repeated measurements, followed by Wilcoxon matched pairs when appropriate) and between surgical groups on a given testing day (Kruskal–Wallis ANOVA, followed by Mann–Whitney U-test when appropriate). A p value of <0.05 was considered to be statistically significant. Data are presented as median with 25th and 75th quartiles.

3. Results

3.1. Mechanical stimulation of the skin overlying a tibial neuroma produces a behavioral response

In experiment one, eight animals underwent the tibial neuroma transposition (TNT) surgery in which the tibial nerve was ligated and rotated such that the ligature was positioned at the lateral side of the ankle (Fig. 3A). The three control groups in experiment one are illustrated in Figs. 3B–D. Mechanical stimulation of the lateral side of the ankle with a von Frey probe normally did not lead to a behavioral response. However, following the TNT surgery, animals developed a vigorous response to von Frey stimulation at the ligature (which could be visualized through the skin). The incidence of response to five trials of mechanical stimulation is plotted as a function of time after the lesion in Fig. 4. The response frequency for the TNT group differed significantly from baseline starting on postoperative day 5 and persisting for the duration of the experiment (100 days). The response frequency for the TNT group differed from the three control groups starting on day six and for most of the time points thereafter.

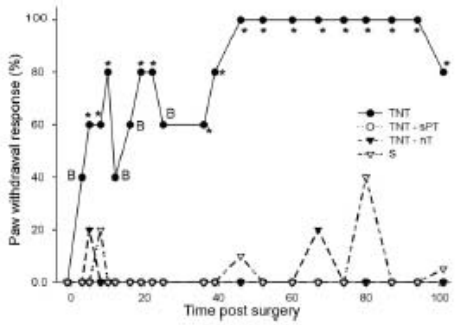


Fig. 4. TNT model produces neuroma tenderness. Following TNT surgery, animals displayed an increased frequency of response to application of a 150 mN von Frey probe to the ligature site. The median behavioral response frequency for the TNT group differed significantly from baseline starting on postoperative day 5 ($B = p < 0.05$). The TNT group differed significantly from the three control groups starting on day 7 ($^{*} = p < 0.05$ with respect to baseline and with respect to other groups). The control groups did not differ significantly from baseline or each other. Schematics of the surgeries performed in each of the groups are shown in Fig. 3.

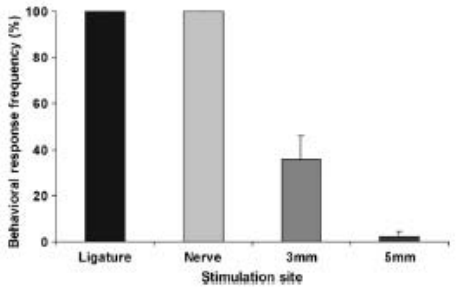


Fig. 5. Focal region of neuroma tenderness in TNT model. The behavioral response frequency to application of a 150 mN von Frey probe was measured at four sites on the lateral hindlimb: the ligature site, 3 mm proximal to the ligature (on the tibial nerve), 3 mm inferior to the ligature, and 5 mm inferior to the ligature ($n = 9$).

There was no consistent difference in response frequency for any of the control groups compared to baseline or each other. These control groups were run concurrently with the TNT model animals to insure blinding of the experimenter. Perhaps the most interesting control group is the TNT-sPT group in which the tibial nerve was ligated and rotated as is done for the TNT model surgery but the tibial nerve was cut simultaneously about 1 cm proximal to the ligature. This group did not display an increased response to mechanical stimulation at the ligature site indicating that the behavior was not due to the surgical manipulations necessary to position the ligature on the lateral side of the foot, but rather require that the nervous supply to the ligature site (and eventual neuroma) was intact.

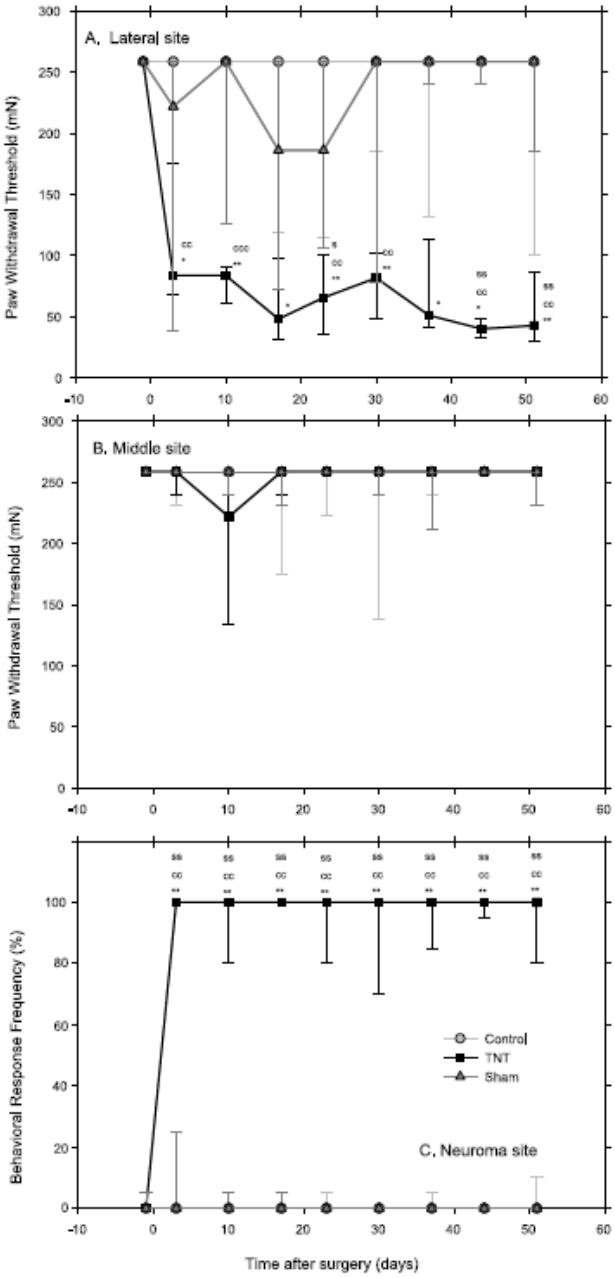
To confirm that this behavior did not reflect cutaneous hyperalgesia but rather required stimulation of the neuroma, we applied the von Frey probe at four different locations relative to the ligature (Fig. 5). Von Frey stimulation to the skin overlying the ligature or along the course of the tibial nerve 3 mm proximal to the ligature always evoked a 100% response in all animals. Response frequencies decreased in a distance dependent manner as the probe was applied 3 mm ($RF = 36 \pm 10\%$) and 5 mm ($RF = 2 \pm 2\%$) inferior to the ligature test site.

3.2. The TNT surgery produces behavioral signs of mechanical hyperalgesia in the hindpaw

Experiment two served as a randomized, controlled assay for the development of mechanical hyperalgesia in the hindpaw following TNT model surgery. Paw withdrawal thresholds to mechanical stimuli applied to the lateral paw and middle paw are shown in Fig. 6. At baseline, there was no difference in withdrawal thresholds at either site amongst the groups. In all groups, the withdrawal thresholds in the middle of the hindpaw (tibial nerve distribution) were at or near the cut off value for all time points, reflecting the fact that the animals did not respond to the highest von Frey before the injury and that the middle of the paw is almost completely denervated by the tibial lesion.

The mechanical withdrawal thresholds in the lateral aspect of the hindpaw varied significantly with group and time. Animals in the TNT model group displayed mechanical withdrawal thresholds that were significantly lower than baseline and the naive control group for the duration of the postoperative period (51 days) with the exception of days 17 and 37 when they were only significantly lower than baseline. The TNT model group displayed paw withdrawal thresholds that tended to be lower than those of the sham group on all postop-

Fig. 6. TNT model produces mechanical hyperalgesia. Paw withdrawal thresholds to von Frey stimuli applied to the lateral (A) and middle (B) test sites are plotted as a function of time after the surgery. (A) At the lateral test site, animals in the TNT model group (filled square, $n = 8$) displayed mechanical withdrawal thresholds that were significantly lower than baseline ($^{*} = p \leqslant 0.05$, $^{**} = p \leqslant 0.01$), the non-operated control (circle, $n = 8$, $^{\circ} = p \leqslant 0.05$, $^{\circ\circ} = p \leqslant 0.01$), and the sham group (triangle, $n = 8$, $^{\#} = p \leqslant 0.05$, $^{\#\#} = p \leqslant 0.01$). There was no difference in withdrawal thresholds at either site amongst the groups at baseline. (B) In all groups, the withdrawal thresholds in the middle of the hindpaw (tibial nerve distribution) did not vary significantly from baseline at any point in the postoperative period. (C) Tenderness over the lateral ankle developed in all animals following TNT model surgery, but not in the sham or control animals. The response frequencies for the TNT model group were significantly elevated compared to non-operated control group ($^{\circ\circ} = p \leqslant 0.01$), the sham group ($^{\#\#} = p \leqslant 0.01$), and baseline ($^{**} = p \leqslant 0.01$).


A. Lateral site
B. Middle site
C. Neuroma site
Paw Withdrawal Threshold (mN)
Paw Withdrawal Threshold (mN)
Behavioral Response Frequency (%)
Time after surgery (days)
Control
TNT
Sham

erative days. This difference reached significance on days 23, 44, and 51. For the sham and naive groups, lateral-site paw withdrawal thresholds did not vary significantly from baseline or each other.

3.3. Proximal tibial nerve transection reverses the neuroma tenderness produced by the TNT model

Experiment three evaluated the effect of a delayed, proximal tibial nerve transection on neuroma tenderness. Twenty of the twenty-four animals that underwent TNT model surgery displayed behavioral response scores that were significantly greater than baseline six days after surgery. Ten days after the TNT model surgery, 11 of these animals underwent proximal tibial nerve transection. The remaining 9 animals had the tibial nerve exposed (but not cut) and served as the control animals to blind the experimenter.

Following proximal tibial nerve transection, behavioral response scores for stimulation at the neuroma dropped abruptly and were not significantly different from the baseline scores before the TNT surgery. The scores were significantly lower than immediately prior to proximal tibial nerve transection for the entire testing period (Fig. 7). In contrast, following tibial nerve exposure in the control group, response scores did not decrease but remained significantly greater than baseline and did not vary significantly from immediately prior to tibial nerve exposure. Behavioral response scores were significantly lower for the proximal tibial nerve transection compared to tibial nerve exposure group on all postoperative test days. Thus, proximal tibial nerve transection led to a reversal of the neuroma pain behavior.

3.4. Mechanical hyperalgesia in the hindpaw produced by the TNT model persists following proximal tibial nerve transection

Experiment three was also used to assess the effects of proximal tibial nerve transection on mechanical hyperalgesia produced by TNT model surgery. At baseline, paw withdrawal thresholds for the two groups did not differ (Fig. 7B). Immediately after TNT model surgery, paw withdrawal thresholds on the lateral side of the foot were significantly lower than baseline for both groups. No difference was evident between the groups. Following proximal tibial nerve exposure or transection, paw withdrawal thresholds remained significantly decreased from baseline for all animals. There was no difference in paw withdrawal threshold between the two groups at any time point. Thus, proximal tibial nerve transection did not lead to a reversal of the hindpaw hyperalgesia.

A total of 40 animals received TNT surgery in the three experimental groups. Thirty-six of these animals (90%) displayed a positive behavioral response to mechanical stimulation of the skin overlying the neuroma.

3.5. Local lidocaine injection reverses the neuroma tenderness produced by the TNT model, but does not effect hindpaw mechanical hyperalgesia

Fig. 8 illustrates the effects of local lidocaine injection on neuroma tenderness and hindpaw mechanical hyperalgesia compared to the effects of lidocaine injection at a remote site. Eight TNT animals from experiment group two that displayed increased behavioral response frequencies at the neuroma-site and hindpaw mechanical hyperalgesia nine weeks after tibial nerve neuroma model surgery were enrolled in a crossover study. Response frequencies were significantly lowered following local, but not remote, lidocaine injection. This was first evident at 15 min and lasted for the duration of the experiment (120 min). Behavioral response frequencies following remote lidocaine injection did not significantly differ from pre-injection levels.

Paw withdrawal thresholds to mechanical stimuli applied to the lateral aspect of the paw did not differ from pre-injection levels following remote or local lidocaine injection. Following injection, the withdrawal thresholds of the two groups did not differ with respect to each other.

3.6. TNT model surgery results in the formation of a histologically characteristic neuroma

Histological sections of the neuroma, proximal nerve and distal nerve stump were examined at the completion of the experiments, some seven months after creation of the neuroma. Longitudinal sections through the neuroma demonstrated demyelination, enlarged unmyelinated axons, increased collagen, excess endoneurial cells and chaotic orientation of axons, all features characteristic of nerve-end neuromas that do not undergo rotation (Fried and Devor, 1988). In the area of the neuroma nearest the ligature, there were numerous, large, unmyelinated axons as previously observed in detailed studies of neuroma endbulb formation (Fried et al., 1991). Unlike previous studies, we observed a significant number of thinly myelinated axons within several hundred microns of the ligature site (Fig. 9), presumably reflecting very late changes in these neuromas studied more than 200 days after nerve ligation. More proximal parts of the nerve, leading to the neuroma, exhibited a normal density of axons and the formation of some regenerative clusters.

The distal nerve stumps that were generated at the time of the initial injury were identified at autopsy by microdissection. Perhaps surprisingly, these distal nerve stumps were not denervated but rather exhibited a large number of axons, although the number was markedly reduced when compared to uninjured nerves. The origin

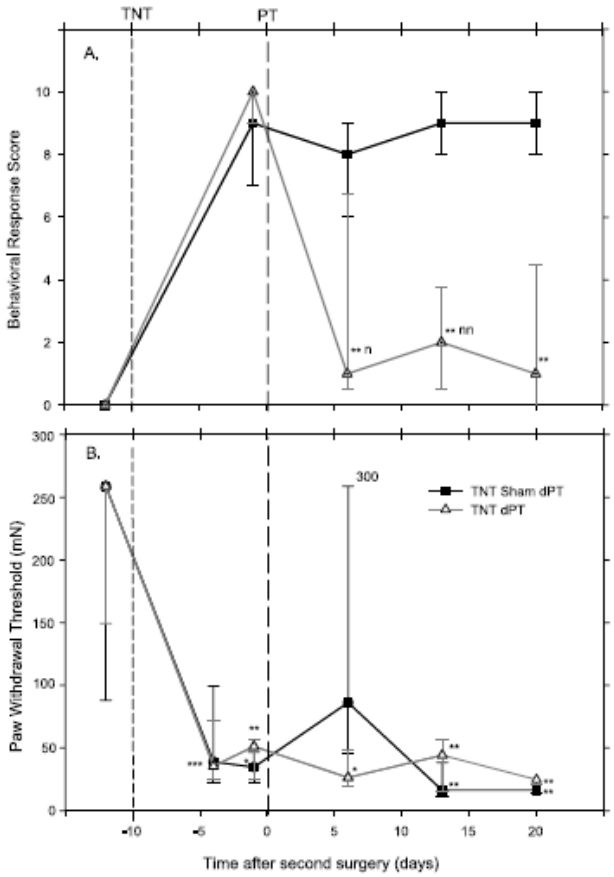


Fig. 7. Proximal tibial nerve transection reverses neuroma tenderness. The TNT model surgery was done on all animals. Ten days later, the tibial nerve was exposed 1 cm proximal to the ligature site in animals that had developed robust pain behaviors ($n = 20$). The nerve was transected in 11 of the animals (TNT-dPT) and left alone in the others (TNT sham dPT). (A) Neuroma tenderness is reversed by proximal tibial nerve transection. The TNT surgery produced behavioral response scores that were significantly greater than baseline for both groups. The delayed proximal transection (dPT), but not sham, resulted in a significant decrease in the behavioral response scores to a level that was not significantly different from baseline. The behavioral response scores remained significantly lower than immediately following TNT model ($^{**} = p \leqslant 0.01$) for the entire testing period. Compared to sham dPT group, the dPT group demonstrated behavioral response scores that were significantly lower on all postoperative test days ($^{n} = p \leqslant 0.05$, $^{nn} = p \leqslant 0.01$). (B) Paw hyperalgesia is not changed by proximal tibial nerve transection. At baseline, paw withdrawal thresholds for the two groups did not differ. Immediately after the TNT surgery, paw withdrawal thresholds were significantly lower than baseline for both groups. No difference was evident between the groups. Following dPT or sham dPT, paw withdrawal thresholds remained significantly decreased from baseline and did not differ from post-TNT model levels ($^{*} = p \leqslant 0.05$, $^{**} = p \leqslant 0.01$, $^{***} = p \leqslant 0.001$). There was no difference in paw withdrawal threshold between the two groups at any time point.

and directionality of these axons was not established in these experiments, and it is possible that these axons were retrograde or anterograde in direction (Belzberg and Campbell, 1998). As described below, we demonstrate that these axons did not arise from the tibial nerve itself.

Proximal tibial nerve transection (Experiment three) resulted in massive Wallerian degeneration in the nerve-end neuromas when examined on day 5 post-transection. Following proximal tibial nerve transection, however, there was no Wallerian degeneration seen in the distal nerve stump indicating that repopulation of

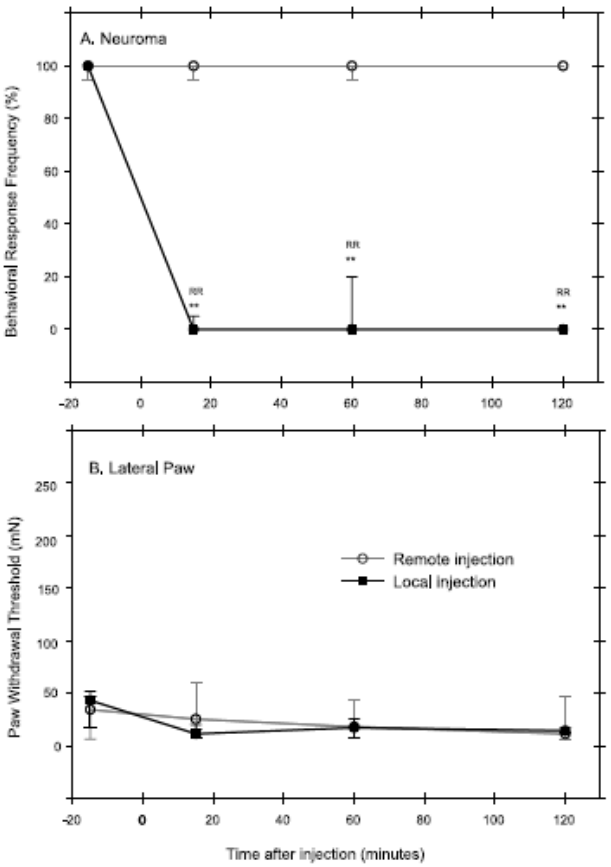


Fig. 8. Lidocaine injection at the site of the neuroma reverses neuroma tenderness. Nine weeks after TNT surgery, lidocaine (1%, 100 μl) was injected at the site of the neuroma or at a remote site. (A) The local lidocaine injection resulted in a significant decrease in the response frequency to mechanical stimulation of the neuroma (** = $p \leqslant 0.01$). Injection of lidocaine to a remote site did not affect the response frequency. The response frequencies were significantly lower following injection for the local versus remote injection group for the entire 120-min test period ($^{RR} = p \leqslant 0.01$). (B) Lidocaine injection, local or remote, did not alter paw withdrawal thresholds to mechanical stimulation of the plantar hindpaw at any time point following injection.

the distal nerve stump was not due to invasion by axons arising from the tibial nerve but rather to recruitment of axons from other nerves. This suggests that reinnervation of the plantar skin may be due in part to axons from adjacent nerves that have regenerated through the distal stump of the tibial nerve.

3.7. Afferent fibers originating from the tibial neuroma exhibited spontaneous activity and mechanosensitivity

In animals with a tibial nerve neuroma ($n = 3$), single fiber recordings were obtained from 130 units (90 C fibers and 40 A fibers) in the tibial nerve. Spontaneous activity was observed in 14 fibers; in two of these fibers the spontaneous activity originated from cold fibers since it was stopped by gentle warming. Mechanical stimulation of the neuroma elicited a response in 5 fibers. In the sham animal, single fiber recordings were obtained from 26 fibers (19 C fibers and 7 A fibers). Spontaneous activity was observed in 2 fibers; both were cold fibers since gentle warming stopped the spontaneous activity. Mechanical stimulation over the nerve did not elicit a response. These results are comparable to those obtained by others who have recorded from neu-

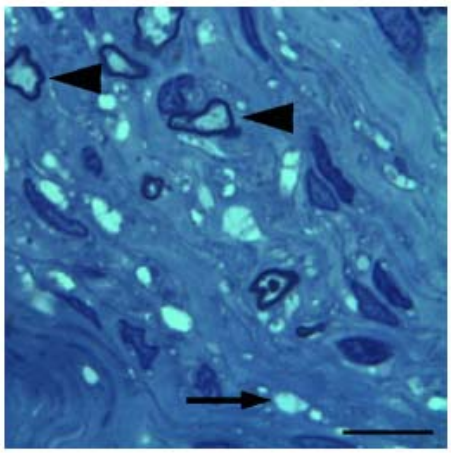


Fig. 9. Thinly myelinated axons in long-term neuromas. Longitudinal sections through a representative neuroma at seven months after surgery demonstrate thinly myelinated axons in both longitudinal and transverse orientation (arrowheads). Large unmyelinated axons, typical of endbulbs, are also seen (arrow). There is an increase in unmyelinated axons, non-neuronal endoneurial cells and collagen. Bar equals 40 μ.

romas in the peripheral nerve (Blumberg and Jänig, 1984; Meyer et al., 1985).

4. Discussion

We developed a novel model of neuropathic pain to specifically investigate the mechanisms of neuroma pain. The tibial neuroma transposition (TNT) model produces a robust and lasting behavioral response characterized by tenderness to mechanical stimuli applied over the neuroma and hyperalgesia to mechanical stimuli applied to the plantar hindpaw. The neural mechanisms of neuroma tenderness and mechanical hyperalgesia appear to be different since interruption of the pain signaling pathway from the neuroma to the CNS by local application of lidocaine or by proximal transection of the tibial nerve eliminates the neuroma tenderness but not the plantar mechanical hyperalgesia.

The basis for the TNT model is the clinical observation that palpating the tissue overlying a neuroma evokes paraesthesias/dysaesthesias in the distribution of the injured nerve. Anecdotal reports indicate that palpating sciatic nerve neuromas in rats evokes distress vocalization and struggling (Devor et al., 1999). The TNT model is the first animal model that allows neuroma tenderness to be systematically evaluated.

4.1. Neuroma tenderness

Several measures were taken to ensure that the behavioral response was dependent on the stimulation of the neuroma. To avoid the possibility that the behavioral response was due to the hyperalgesia often seen in the distribution of an injured nerve or due to incisional pain, the tibial neuroma was rotated from its natural position to the lateral aspect of the hindlimb. Control groups for the different surgical steps in the TNT model were used to exclude behaviors associated with surgically damaged soft tissue. Maps of the area of mechanical hypersensitivity demonstrated that the evoked behavior was specific to stimuli applied to the neuroma and not to hyperalgesia of the adjacent tissue. Finally, we were able to reverse the neuroma tenderness by interrupting signaling from the neuroma to the CNS.

The severity, robustness, and duration of neuropathic pain behaviors produced by the TNT model are comparable to those of other models of neuropathic pain including the chronic constriction injury (Bennett and Xie, 1988), partial sciatic nerve ligation (Seltzer et al., 1990), spinal nerve ligation (Kim and Chung, 1992), and a spared nerve injury (Decosterd and Woolf, 2000). The neuroma tenderness appeared within several days.

Within hours of a nerve transection, ectopic mechanosensitivity develops at the severed nerve tips (Welk et al., 1990; Koschorke et al., 1991; Michaelis et al., 1995). In myelinated fibers, the incidence of mechanically sensitivity increases over the first 24 h reaching a level of about 25%. This is presumably due to the axonal transport and accumulation of transduction elements at the severed tip (Koschorke et al., 1994). For unmyelinated fibers, the incidence of mechanosensitivity is about 13% and remains relatively constant over a 2-month period (Welk et al., 1990). Afferent fibers whose regenerating sprouts become trapped in neuromas also develop ectopic spontaneous activity, crosstalk, and sensitivity to thermal and chemical stimuli (Blumberg and Jänig, 1984; Devor et al., 1999; Michaelis et al., 1999; Rivera et al., 2000). Our findings of spontaneous activity and mechanically-evoked responses in A-fiber and C-fiber afferents in the tibial neuromas are consistent with these reports.

The ectopic mechanosensitivity of afferents trapped in the neuroma is believed to be responsible for the abnormal sensory phenomena evoked by neuroma palpation. Microneurography in a patient with a peroneal nerve neuroma revealed that percussion of the neuroma elicited an intense burst of spike activity and augmentation of the patient's pain (Nyström and Hagbarth, 1981). In experimental neuroma preparations, 'hot spots' of mechanosensitivity are clustered at the nerve endbulb (Devor et al., 1999). Following a crush injury or nerve section with resuturing, mechanosensitive sites

have been observed up to 6 mm proximal to the injury site (Gorodetskaya et al., 2003) presumably due to retrograde sprouting. Our finding that mechanical stimulation of the tibial nerve trunk proximal to the neuroma evoked a behavioral response is consistent with the existence of mechanosensitive spots proximal to the injury site.

Changes in the phenotype, quantity, and distribution of ion channels (specifically sodium channels that accumulate in the nerve stump) may underlie the ectopic electrical properties that arise in injured afferents (Devor et al., 1999). Systemic or topical application of a range of "membrane stabilizers" (Na channel blockers) rapidly silences abnormal firing generated at nerve-injury sites in rats (Yaari and Devor, 1985; Burchiel, 1988; Devor et al., 1994; Matzner and Devor, 1994). Further, perioneuromal or trigger point injections of local anesthetic often provide relief for patients with painful neuromas (Chabal et al., 1992; Gracely et al., 1992). Consistent with this latter observation is our finding that the neuroma tenderness is reversed by local lidocaine injection.

4.2. Plantar hyperalgesia

Following nerve injury, patients report allodynia and hyperalgesia in the partly denervated skin (Trotter and Davies, 1909; Sunderland, 1978; Fishbain et al., 1996; Bonica et al., 2001). An advantage of the TNT model is that it produces both mechanical hyperalgesia in the cutaneous territory of the sural nerve and neuroma tenderness. The sural nerve territory lies adjacent to and partially overlaps the denervated tibial nerve territory (Swett and Woolf, 1985). Similarly, lesion of two of three terminal branches of the sciatic nerve (tibial and peroneal) also produces robust mechanical hyperalgesia in the cutaneous territories of the spared sural and saphenous nerves (Decosterd and Woolf, 2000).

In the present study, proximal tibial nerve transection reversed neuroma tenderness, but not plantar mechanical hyperalgesia. This supports the hypothesis that the two behaviors have distinct mechanisms. The neuroma tenderness is dependent on activity originating from the neuroma. The persistence of plantar mechanical hyperalgesia suggests that this hyperalgesia is independent of ectopic activity from the neuroma.

The plantar hyperalgesia seen in this model is similar to that seen in other neuropathic models involving traumatic nerve injuries and is likely due to similar mechanisms. Many authors think that hyperalgesia is a result of central sensitization to input from normal afferents. What drives this central sensitization is controversial. Ectopic activity from injured afferents appears to play a role in some studies (Liu et al., 2000). Another possibility is that adjacent, uninjured nociceptive afferents develop spontaneous activity (Wu et al., 2001) that might drive central sensitization. A recent study reported that uninjured nociceptors become sensitized to mechanical stimuli after a spinal nerve ligation injury (Shim et al., 2005), and therefore central sensitization may not be required.

Since behavioral testing was not performed until several days after the proximal tibial nerve transection, we cannot exclude the possibility that a new neuroma developed at the transection site and became the focus of ectopic impulse generation that could drive the central sensitization responsible for the plantar hyperalgesia. For example, spontaneous activity in unmyelinated afferents can develop within 30 h of nerve section (Michaelis et al., 1995). However, lidocaine injections at the neuroma site reversed the neuroma tenderness (and presumably ectopic activity from the injury site) but also did not reverse the hyperalgesia. These manipulations did not block ectopic impulses in injured afferents that may arise from more proximal locations along the nerve trunk or the DRG.

4.3. Ongoing pain

Patients describe ongoing burning, cramping, or lancinating sensations in the distribution of the injured nerve. Ongoing pain may be due, at least in part, to movement of the neuroma which is tethered to adjacent tissue since surgical repositioning of the neuroma to minimize movement can alleviate some of the pain. Measurements of spontaneous pain in animals have been problematic. Several authors have advocated that self-mutilating behavior, termed autotomy, observed after sciatic nerve transection is an indication of spontaneous pain (Wall et al., 1979; Levitt, 1985; Coderre et al., 1986; Blumenkopf and Lipman, 1991; Seltzer et al., 1991). Others argue that the autotomy behavior represents a reaction to chronic paraesthesias, excessive grooming, or a proclivity of some species to shed a functionally impaired insensate limb (Rodin and Kruger, 1984; Lindblad and Ekenvall, 1986; Moossy et al., 1987). Although the TNT model produces ectopic electrical activity and stimulus evoked pain behaviors, none of the animals exhibited autotomy. Other forms of spontaneous pain behaviors (e.g., hindlimb flinching, scratching, or biting) were not observed. Therefore, the presence and/or degree of spontaneous pain produced by the TNT model remains uncertain.

4.4. Clinical relevance

Many of the drugs currently used to treat neuropathic pain result in unacceptable side effects such as sedation and cognition impairment. The TNT model will be an important tool in the preclinical development of new therapies for neuropathic pain. The TNT model allows neuroma tenderness to be investigated indepen-

dent of hyperalgesia. This provides the opportunity to investigate novel therapeutic strategies that specifically target neuroma pain.

Acknowledgments

We greatly appreciate the assistance of Timothy V. Hartke and Yehuda Belzberg. This research was funded in part by a grant from the US Army Medical Research and Materiel Command (W81XWH-06-1-0176) and the Johns Hopkins Blaustein Pain Research Fund.

References

Belzberg AJ, Campbell JN. Evidence for end-to-side sensory nerve regeneration in a human. J Neurosurg 1998;89:1055–7.

Bennett GJ, Xie Y-K. A peripheral mononeuropathy in rat that produces disorders of pain sensation like those seen in man. Pain 1988;33:87–107.

Blumberg H, Jänig W. Discharge pattern of afferent fibers from a neuroma. Pain 1984;20:335–53.

Blumenkopf B, Lipman JL. Studies in autotomy: its pathophysiology and usefulness as a model of chronic pain. Pain 1991;45:203–9.

Bonica JJ, Liebeskind JC, Albe-Fessard D. Causalgia and other reflex sympathetic dystrophies. In: Bonica JJ, editor. Advances in pain research and therapy 2001;vol. 3. New York: Raven Press; 2001. p. 141–66.

Burchiel KJ. Carbamazepine inhibits spontaneous activity in experimental neuromas. Exp Neurol 1988;102:249–53.

Campbell JN, Meyer RA. Sensitization of unmyelinated nociceptive afferents in the monkey varies with skin type. J Neurophysiol 1983;49:98–110.

Chabal C, Jacobson L, Russell LC, Burchiel KJ. Pain response to perineuromal injection of normal saline, epinephrine, and lidocaine in humans. Pain 1992;49:9–12.

Coderre TJ, Grimes RW, Melzack R. Deafferentation and chronic pain in animals: an evaluation of evidence suggesting autotomy is related to pain. Pain 1986;26:61–84.

Decosterd I, Woolf CJ. Spared nerve injury: an animal model of persistent peripheral neuropathic pain. Pain 2000;87:149–58.

Devor M, Lomazov P, Matzner O. Sodium channel accumulation in injured axons as a substrate for neuropathic pain. In: Boivie J, Hansson P, Lindblom U, editors. Touch, temperature, and pain in health and disease: mechanisms and assessments. Seattle: IASP Press; 1994. p. 207–30.

Devor M., Seltzer Z. Pathophysiology of damaged nerves in relation to chronic pain. In: Wall PD, Melzak R, editors. Textbook of pain. Edinburgh, London, New York, Philadelphia, St. Louis, Sydney, Toronto: Churchill Livingstone; 1999. p. 129–64.

Dixon WJ. Efficient analysis of experimental observations. Ann Rev Pharmacol Toxicol 1980;20:441–62.

Eschenfelder S, Häbler H-J, Jänig W. Dorsal root section elicits signs of neuropathic pain rather than reversing them in rats with L5 spinal nerve injury. Pain 2000;87:213–9.

Fishbain DA, Chabal C, Abbott A, Heine LW, Cutler R. Transcutaneous electrical nerve stimulation (TENS) treatment outcome in long-term users. Clin J Pain 1996;12:201–14.

Fried K, Devor M. End-structure of afferent axons injured in the peripheral and central nervous system. Somatosens Mot Res 1988;6:79–99.

Fried K, Govrin-Lippmann R, Rosenthal F, Ellisman MH, Devor M. Ultrastructure of afferent axon endings in a neuroma. J Neurocytol 1991;20:682–701.

Gerodetskaya N, Constantin C, Jänig W. Ectopic activity in cutaneous regenerating afferent nerve fibers following nerve lesion in the rat. Eur J Neurosci 2003;18:2487–97.

Gracely RH, Lynch SA, Bennett GJ. Painful neuropathy: altered central processing maintained dynamically by peripheral input. Pain 1992;51:175–94.

Kim SH, Chung JM. An experimental model for peripheral neuropathy produced by segmental spinal nerve ligation in the rat. Pain 1992;50:355–63.

Koschorke GM, Meyer RA, Tillman DB, Campbell JN. Ectopic excitability of injured nerves in monkey: entrained responses to vibratory stimuli. J Neurophysiol 1991;65:693–701.

Koschorke G-M, Meyer RA, Campbell JN. Cellular components necessary for mechanoelectrical transduction are conveyed to primary afferent terminal by fast axonal transport. Brain Res 1994;641:99–104.

Levitt M. Dysesthesias and self-mutilation in humans and subhumans: a review of clinical and experimental studies. Brain Res 1985;357:247–90.

Li Y, Dorsi MJ, Meyer RA, Belzberg AJ. Mechanical hyperalgesia after an L5 spinal nerve lesion in the rat is not dependent on input from injured nerve fibers. Pain 2000;85:493–502.

Lindblad LE, Ekenvall L. Alpha-adrenoceptors in the vessels of human finger skin. Acta Physiol Scand 1986;128:219–22.

Liu C-N, Wall PD, Ben Dor E, Michaelis M, Amir R, Devor M. Tactile allodynia in the absence of C-fiber activation: altered firing properties of DRG neurons following spinal nerve injury. Pain 2000;85:503–21.

Matzner O, Devor M. Hyperexcitability at sites of nerve injury depends on voltage-sensitive Na^+ channels. J Neurophysiol 1994;72:349–59.

Meyer RA, Raja SN, Campbell JN, Mackinnon SE, Dellon AL. Neural activity originating from a neuroma in the baboon. Brain Res 1985;325:255–60.

Michaelis M, Blenk KH, Vogel C, Jänig W. Distribution of sensory properties among axotomized cutaneous C-fibres in adult rats. Neuroscience 1999;94:7–10.

Michaelis M, Blenk K-H, Jänig W, Vogel C. Development of spontaneous activity and mechanosensitivity in axotomized afferent nerve fibers during the first hours after nerve transection in rats. J Neurophysiol 1995;74:1020–7.

Moossy JJ, Nashold Jr BS, Osborne D, Friedman AH. Conus medullaris nerve root avulsions. J Neurosurg 1987;66:835–41.

Nyström B, Hagbarth K-E. Microelectrode recordings from transected nerves in amputees with phantom limb pain. Neurosci Lett 1981;27:211–6.

Ringkamp M, Grethel EJ, Choi Y, Meyer RA, Raja SN. Mechanical hyperalgesia after spinal nerve ligation in rat is not reversed by intraplantar or systemic administration of adrenergic antagonists. Pain 1999;79:135–41.

Rivera L, Gallar J, Pozo MA, Belmonte C. Responses of nerve fibres of the rat saphenous nerve neuroma to mechanical and chemical stimulation: an in vitro study. J Physiol 2000;527 Pt 2:305–13.

Rodin BE, Kruger L. Deafferentation in animals as a model for the study of pain: an alternative hypothesis. Brain Res 1984;319:213–28.

Seltzer Z, Dubner R, Shir Y. A novel behavioral model of neuropathic pain disorders produced in rats by partial sciatic nerve injury. Pain 1990;43:205–18.

Seltzer Z, Paran Y, Eisen A, Ginzburg R. Neuropathic pain behavior in rats depends on the afferent input from nerve-end neuroma including histamine-sensitive C-fibers. Neurosci Lett 1991;128:203–6.

Sheth RN, Dorsi MJ, Li Y, Murinson BB, Belzberg AJ, Griffin JW, et al. Mechanical hyperalgesia after an L5 ventral rhizotomy or an L5 ganglionectomy in the rat. Pain 2002;96:63–72.

Shim B, Kim DW, Kim BH, Nam TS, Leem JW, Chung JM. Mechanical and heat sensitization of cutaneous nociceptors in rats with experimental peripheral neuropathy. Neuroscience 2005;132:193–201.

Sunderland S. Nerves and nerve injuries. New York: Churchill Livingstone; 1978. pp. 120–2.

Swett JE, Woolf CJ. The somatotopic organization of primary afferent terminals in the superficial laminae of the dorsal horn of the rat spinal cord. J Comp Neurol 1985;231:66–77.

Trotter W, Davies HM. Experimental studies in the innervation of the skin. J Physiol 1909;28:134–246.

Wall PD, Devor M, Inbal R, Scadding JW, Schonfeld D, Seltzer Z, et al. Autotomy following peripheral nerve lesions: experimental anaesthesia dolorosa. Pain 1979;7:103–11.

Welk E, Leah JD, Zimmermann M. Characteristics of A- and C-fibers ending in a sensory nerve neuroma in the rat. J Neurophysiol 1990;63:759–66.

Wu G, Ringkamp M, Hartke TV, Murinson BB, Campbell JN, Griffin JW, et al. Early onset of spontaneous activity in uninjured C-fiber nociceptors after injury to neighboring nerve fibers. J Neurosci 2001;21:RC140.

Yaari Y, Devor M. Phenytoin suppresses spontaneous ectopic discharge in rat sciatic nerve neuromas. Neurosci Lett 1985;58:117–22.

SUPPORTING DATA

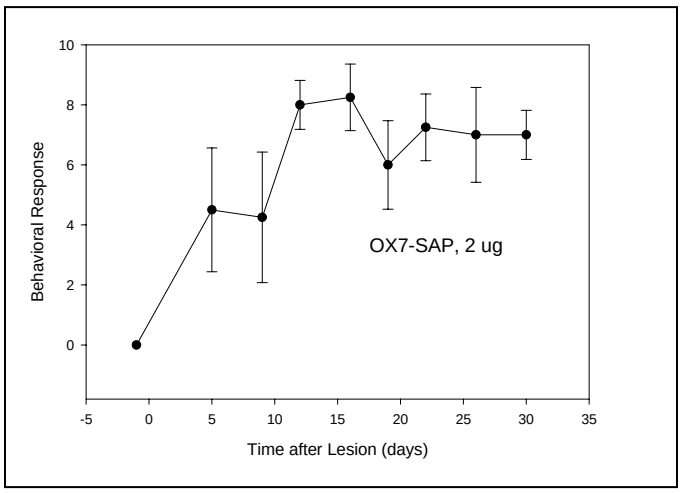


Figure 1 Behavioral responses after OX7-SAP

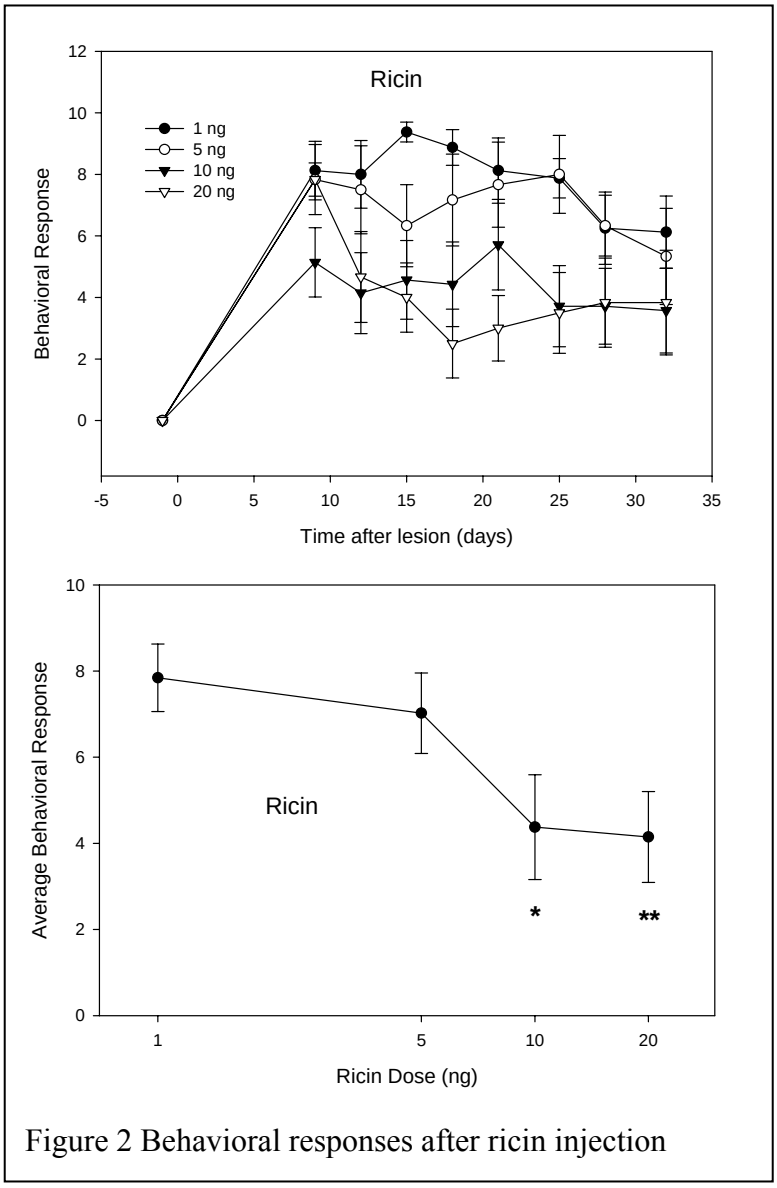


Figure 2 Behavioral responses after ricin injection